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Award Number:

W81XWH-11-1-0181

TITLE:

Mining the Immune Cell Proteome to Identify Ovarian Cancer-Specific Biomarkers

PRINCIPAL INVESTIGATOR:

Dr. Manish S. Patankar

CONTRACTING ORGANIZATION:

University of Wisconsin System
Madison, WI 53715

REPORT DATE:

March 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-03-2012	Annual Report	15 FEB 2011 - 14 FEB 2012

4. TITLE AND SUBTITLE
Mining the Immune Cell Proteome to Identify Ovarian Cancer-Specific Biomarkers

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0181

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Manish S. Patankar

E-Mail: patankar@wisc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Wisconsin System
Madison, WI 53715

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT In previous studies we have demonstrated that the ovarian cancer antigen CA125 specifically binds to certain sunsets of immune cell. Based on these observations we have hypothesized that proteomic and transciptomic analysis of immune cells from ovarian cancer patients will result in the identification of specific biomarkers in the immune cells. The current proposal will further investigate this hypothesis by conducting in-depth proteomic analysis of immune cells from cancer patients and healthy blood donors. Studies conducted have resulted in development of streamlined protocols for proteomic analysis of human immune cells. Proteomic analysis of human NK cells has been completed. In addition to proteomic analysis we have also compared the transcriptome of immune cells from ovarian cancer patients and healthy donors and have identified approximately 1600 genes that are differentially expressed in the patient samples. On-going research is focused on validating the proteomic and transcriptome data and demonstrating changes immune cells in response to cancer antigens.

15. SUBJECT TERMS
Ovarian cancer, immune cells, biomarker, early detection, proteomics, microarray, transcriptome

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	24	19b. TELEPHONE NUMBER *(include area code)*

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Introduction: The primary goal of this project is to identify biomarkers present in immune cells that can be used for the early detection and monitoring of ovarian cancer. Immune cells are continuously exposed to self and cancer antigens. These interactions result in alterations in the transcriptome and proteome of the immune cells. We are therefore proposing that the immune cells contain disease-specific biomarkers. In the current project we proposed to carry out in-depth proteomic analysis to identify the ovarian cancer-specific biomarkers.

Body: Our studies are focused on three specific aims. In the first aim we are conducting experiments to clearly define the proteome of T cells, NK cells, B cells and monocytes. In the second aim we will identify specific changes in the proteomes of immune cells in response to defined antigens derived from ovarian cancer cells. Finally, in the third specific aim experiments will be conducted to compare the proteomes of immune cells isolated from peripheral blood and peritoneal fluid of ovarian cancer patients.

We have now developed streamlined protocols using NKL cells that have allowed us to conduct bottom-up proteomics to identify the proteome of immune cells. Using these streamlined protocols, we have now completed the analysis of human NK cells. This analysis has allowed us to identify approximately 2000 individual proteins in the proteome of human NK cells. This analysis is now complete and we are currently preparing a manuscript on our results that will be submitted to Journal of Proteome research. These experiments address the objectives of Specific Aim 1.

Next we have also obtained data on the proteome of immune cells that are exposed IL-2. These experiments were structured to provide initial data that would show the effect on immune proteomes of factors that the immune cells are likely to encounter in the tumor microenvironment. It is expected that the infiltrating immune cells will likely encounter IL-2. Characterization of the NK cell proteome in response to IL-2 has allowed us to identify not only the proteins that are known to be altered in response to this cytokine but also led to characterization of cellular pathways that have not been classically associated with IL-2 stimulation. Supporting data provided in Figs. 1-6 show the differential expression of proteins in naïve and IL-2 stimulated NK cells. One of the pathways that shows major changes in the expression of NK cell proteins after IL-2 activation, is the JAK/STAT pathway. IL-2 is known to modulate this signaling pathway. While additional validation studies are currently underway, the identification of proteins in the JAK/STAT pathway that are modulated following IL-2 stimulation gives us great confidence in our proteomic analysis of the NK cells.

In addition to conducting the proteomic analysis we have also expanded our studied to include transcriptomic analysis of immune cells. This expansion of the project has been made possible with the availability of microarray chips that are reasonably priced. Taking advantage of these new developments we have now compared the transcriptomic analysis of immune cells isolated from the peripheral blood of healthy donors and ovarian cancer patients. This analysis has been completed in the past month and we are intensely analyzing the data. Our initial analysis has allowed us to identify an approximately 1,600 genes that are either upregulated or downregulated in immune cells from ovarian cancer

patients as compared to those isolated from the peripheral blood of healthy donors. Fig. 7 shows our initial analysis. We are conducting additional exhaustive studies to validate the microarray data and are in the process of identifying gene signatures that can be used as biomarkers for the identification of ovarian cancer-specific biomarkers.

Finally we have also demonstrated that MUC16 (CA125) an antigen produced by ovarian tumors and by the decidua binds to immune cells. After screening samples from ovarian cancer patients, healthy donors, healthy pregnant women and patients with preeclampsia we have found that in each of these cohorts, MUC16 binds to distinct subsets of immune cells. These results provide further proof to our hypothesis that immune cells should be considered as repositories for disease-specific biomarkers. A manuscript on this topic is now published and the support provided by the DOD grant is duly acknowledged.

Key Accomplishments:

1. Streamlined protocols for proteomic analysis of human immune cells have been established.
2. Human NK cell proteome characterization has led to identification of many important proteins.
3. We have identified novel proteins that are differentially expressed in NK cells in response to IL-2
4. Transcriptomic analysis of immune cells from ovarian cancer patients has allowed us to identify genes that are differentially expressed. Efforts are underway to determine a gene expression pattern that can serve as biomarker for ovarian cancer.

Reportable outcomes.

1. A manuscript titled "The Mucin MUC16 (CA125) Binds to NK Cells and Monocytes from Peripheral Blood of Women with Healthy Pregnancy and Preeclampsia" is now published in American Journal for Reproductive Immunology. This manuscript is provided in Appendix XX.
2. Streamlined protocols developed by our group will serve as an excellent source for characterization of the immune cell proteomes in future studies.
3. A graduate student (Ms. Di Ma) supported by the grant is working on the proteomic analysis and will be defending her PhD thesis in the Fall 2012.
4. The transcriptome analysis of immune cells from ovarian cancer patients is being conducted by Dr. Shitanshu Uppal who is undertaking his Gynecologic Oncology fellowship training in our institution. The transcriptome identification project will serve as Dr. Uppal's fellowship research project.

Conclusions. The proteomic, transcriptome analysis and MUC16 research have collectively provided strong evidence supporting the presence of biomarkers for ovarian cancer in circulating immune cells. Our ongoing studies will be focused on consolidating and verifying these initial promising results with the intent of developing novel diagnostic assays for ovarian cancer.

Supporting data

Figs. 1-6: Proteomic analysis of naïve and IL-2 stimulated human NK cells

Fig. 7: Transcriptomic analysis of immune cells from healthy donors versus ovarian cancer patients

Fig. 1. How to choose proteins of interest?

- Pathway analysis help to select the proteins of interest

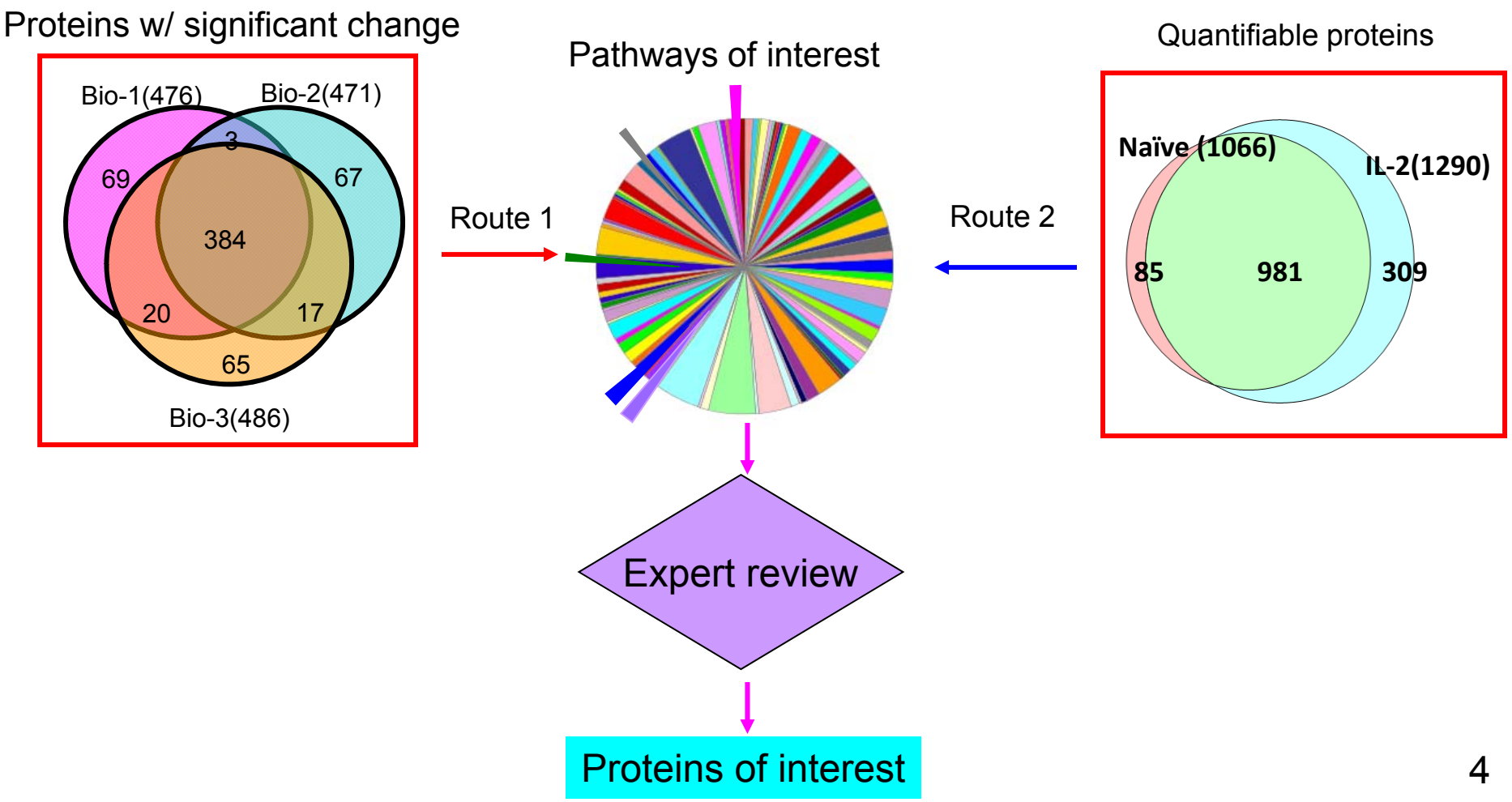
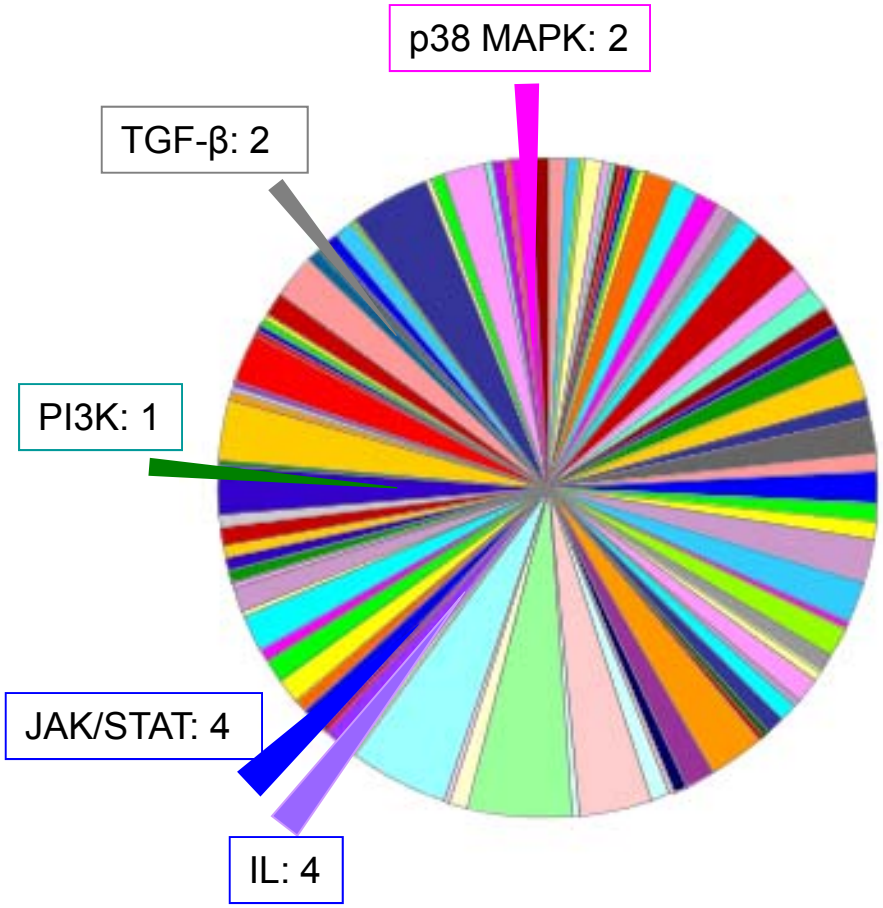


Fig. 2. Proteins of interest in donor 1

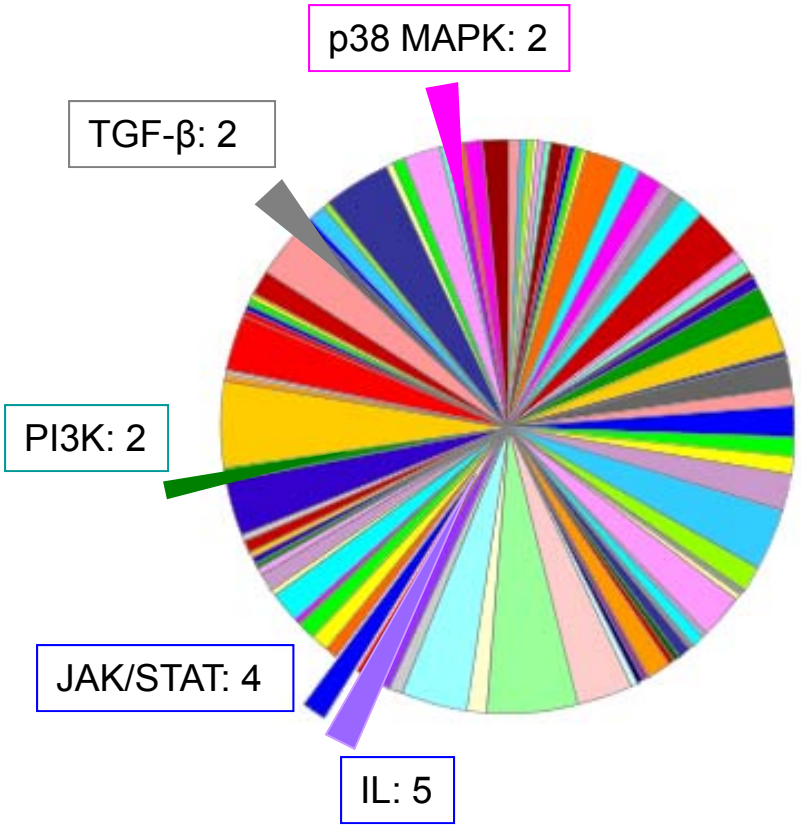


Total 334 pathways found

JAK/STAT	MK14_HUMAN
	STAT1_HUMAN
	STAT3_HUMAN
	STAT4_HUMAN
TGF- β	FKBP2_HUMAN
	MK14_HUMAN
PI3K	GBB2_HUMAN
p38 MAPK	RAC3_HUMAN
	MK14_HUMAN
IL	KS6A3_HUMAN
	STAT1_HUMAN
	STAT4_HUMAN
	STAT3_HUMAN

Proteins of interest: 8

Fig. 3. Proteins of interest in donor 2



Total 289 pathways found

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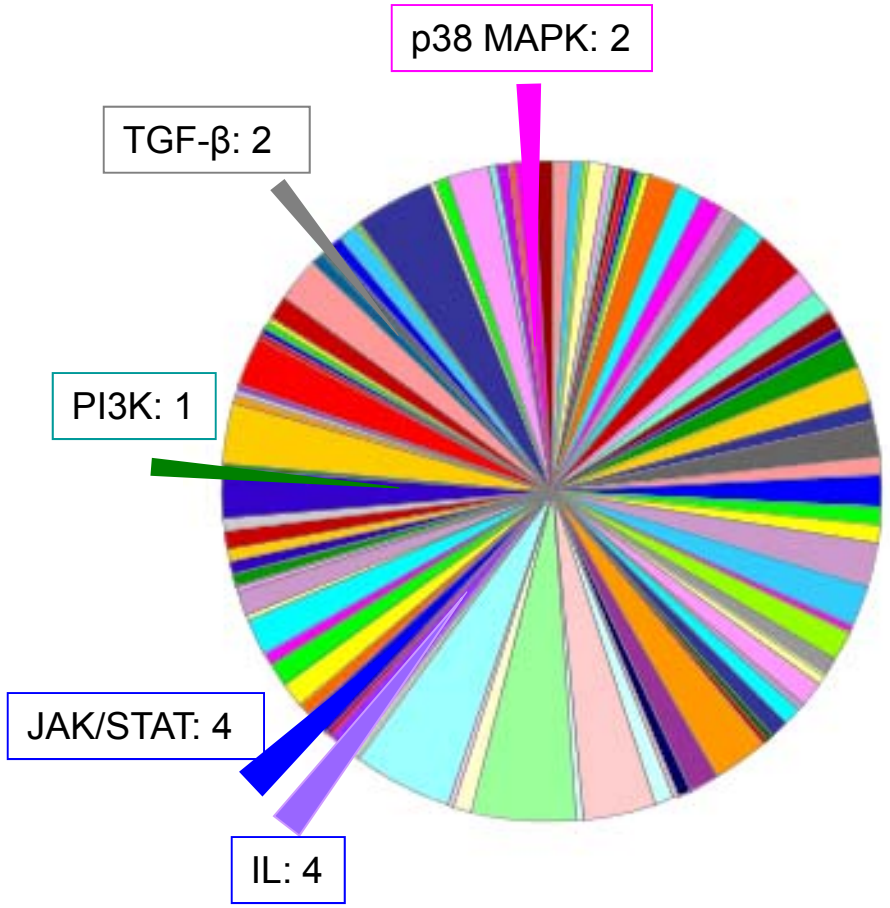
	STAT1_HUMAN
	STAT3_HUMAN
	STAT4_HUMAN
TGF- β	FKBP2_HUMAN
	MK14_HUMAN
PI3K	GRAP2_HUMAN
	1433B_HUMAN
p38 MAPK	RAC3_HUMAN
	MK14_HUMAN
IL	KS6A3_HUMAN
	GRAP2_HUMAN
	STAT1_HUMAN
	STAT3_HUMAN
	STAT4_HUMAN

Proteins of interest: 9

Fig. 4. Proteins of interest in donor 3

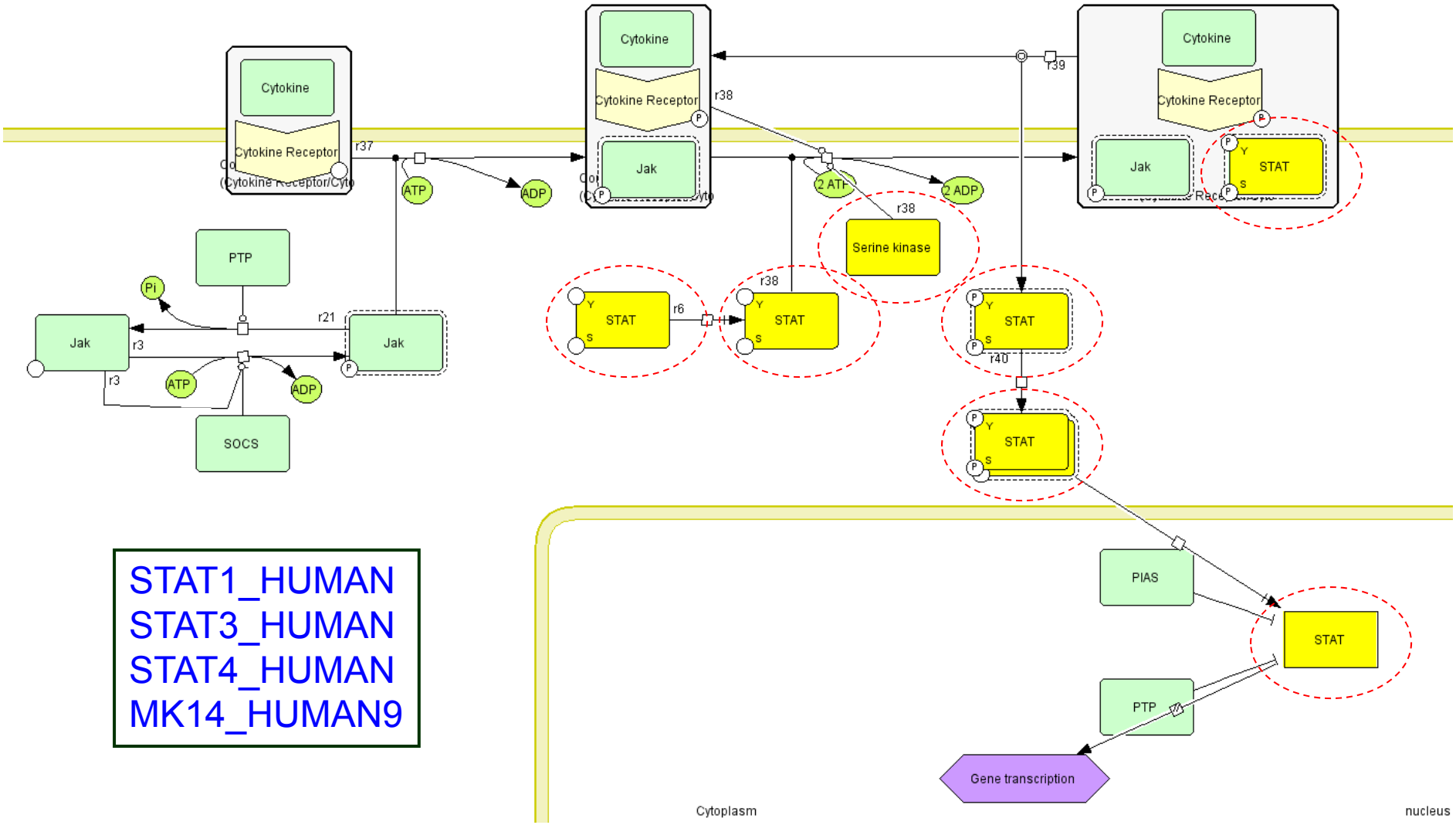


Total 284 pathways found

JAK/STAT	MK14_HUMAN
	STAT1_HUMAN
	STAT3_HUMAN
	STAT4_HUMAN
TGF- β	FKBP2_HUMAN
	MK14_HUMAN
PI3K	1433S_HUMAN
p38 MAPK	RAC3_HUMAN
	MK14_HUMAN
IL	KS6A3_HUMAN
	STAT1_HUMAN
	STAT3_HUMAN
	STAT4_HUMAN

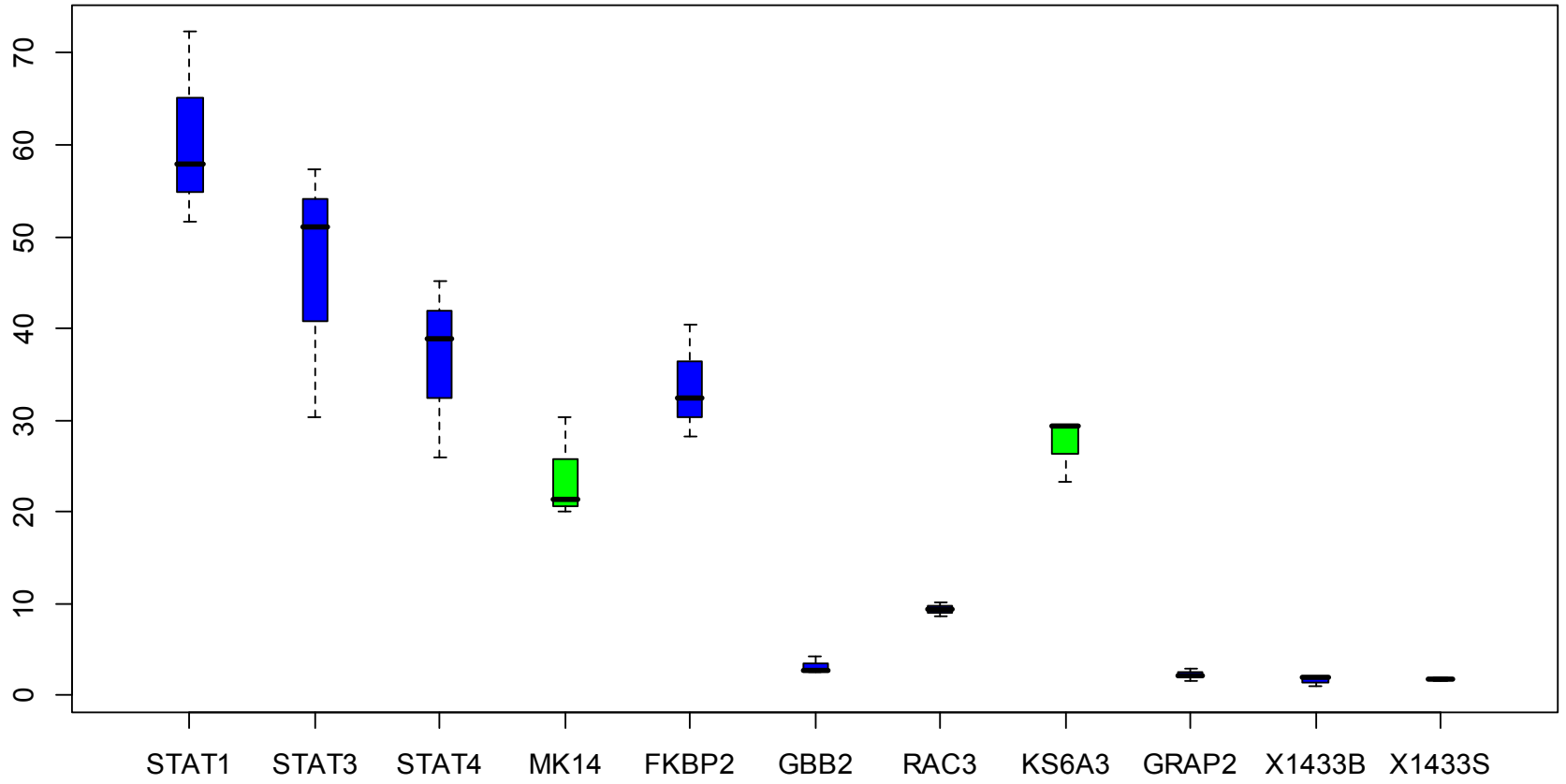
Proteins of interest: 8

Fig. 5. JAK/STAT signaling pathway



STAT1_HUMAN
STAT3_HUMAN
STAT4_HUMAN
MK14_HUMAN9

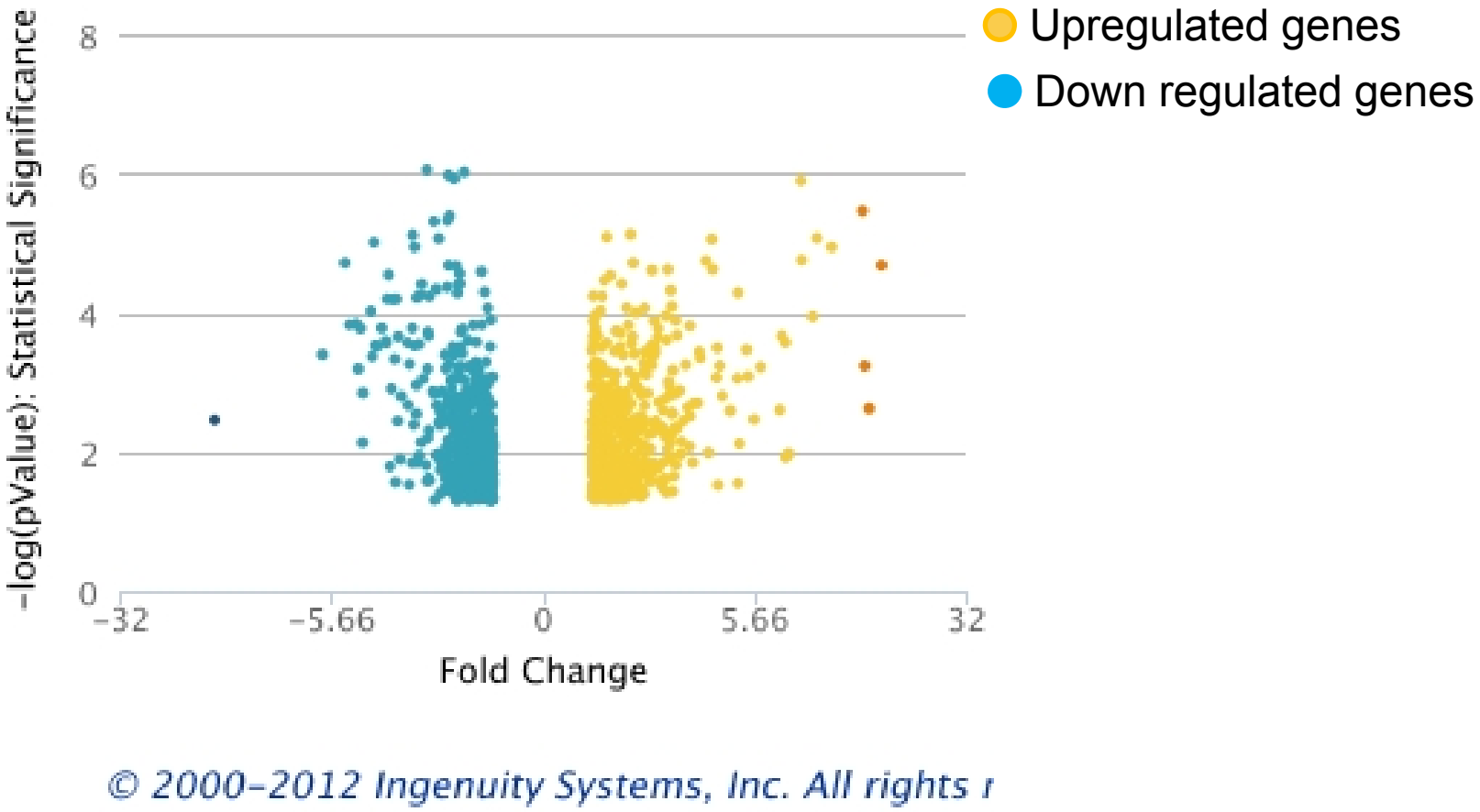
Fig. 6. Selected proteins for validation



Blue – up-regulated
Green – down-regulated

In total, 11 proteins are selected for biological validation

Fig. 7. Differential gene expression in immune cells of ovarian cancer patients



Appendix 1.

The Mucin MUC16 (CA125) Binds to NK Cells and Monocytes from Peripheral Blood of Women with Healthy Pregnancy and Preeclampsia

Chanel Tyler[1], Arvinder Kapur[1], Mildred Felder[1], Jennifer A. Belisle[1], Christine Trautman[1], Jennifer A. A. Gubbels[2], Joseph P. Connor[1], Manish S. Patankar[1]

[1]Department of Obstetrics and Gynecology, University of Wisconsin-Madison, Madison, WI, USA;
[2]Department of Biology, Augustana College, Sioux Falls, SD, USA

Keywords
Biomarker, CA125, immune cell subsets, MUC16, Siglec-9

Correspondence
Manish S. Patankar, Department of Obstetrics and Gynecology, University of Wisconsin-Madison, H4/657 CSC, 600 Highland Avenue, Madison, WI 53792-6188, USA.
E-mail: patankar@wisc.edu

Submission December 7, 2011;
accepted January 30, 2012.

Citation
Tyler C, Kapur A, Felder M, Belisle JA, Trautman C, Gubbels JAA, Connor JP, Patankar MS. The mucin MUC16 (CA125) binds to NK cells and monocytes from peripheral blood of women with healthy pregnancy and preeclampsia. Am J Reprod Immunol 2012

doi:10.1111/j.1600-0897.2012.01113.x

Problem
MUC16 (CA125) released from ovarian tumors binds to NK cells and monocytes via the inhibitory receptor Siglec-9. Here, we investigate whether MUC16 also binds to circulating immune cells during pregnancy and in women with preeclampsia.

Method of study
MUC16 binding was monitored by flow cytometry and immunoprecipitation, and RT-PCR was used to monitor indigenous expression in immune cells. Serum CA125 levels were measured by a clinical assay.

Results
MUC16 was equally distributed on Siglec-9^{pos} CD16^{pos}/CD56^{dim} and CD16^{neg}/CD56^{br} NK cells in the healthy pregnant and preeclampsia groups. While serum CA125 levels and number of NK and monocytes were similar, increased binding of MUC16 was observed on these immune cells in the preeclampsia cohort as compared to the healthy pregnant samples.

Conclusion
MUC16 binding to NK cells and monocytes likely contributes to tolerance of the fetal allograft from maternal responses and may also serve as a novel biomarker for preeclampsia.

Introduction

CA125 is a tumor biomarker used extensively to monitor epithelial ovarian cancer.[1–3] CA125 is a repeating peptide epitope present in the tandem repeat region of MUC16, a 3–5 million Da heavily glycosylated mucin overexpressed by epithelial ovarian tumors.[4–6] We have previously demonstrated that ovarian tumors utilize MUC16 to attenuate the cytolytic responses of NK cells.[7,8] The large molecular weight and high negative charge of this heavily glycosylated mucin also act as a barrier that prevents the NK cells from forming activating immunologic synapses with the ovarian tumor targets.[6,7,9]

MUC16 is a membrane-spanning mucin that is initially expressed on the surface of epithelial cells and especially on the epithelial ovarian tumor cells.[6,10] Proteolytic cleavage results in release of the mucin from the cell surface. The shed mucin (sMUC16) molecules traverse to the peripheral blood from the extracellular milieu, where they can be detected using the clinical serum CA125 test. In our analysis of peripheral blood mononuclear cells (PBMC) isolated from ovarian cancer patients and

the immune cells isolated from their peritoneal fluid (PFMC), we observed that approximately 10–15% of B cells, 30–40% of NK cells, and >90% of monocytes were positive for sMUC16.[11,12] Several lines of evidence indicate that the PBMC and PFMC do not express sMUC16 but instead specifically bind to the mucin released from ovarian tumors.[11,12]

We have now also demonstrated that sMUC16 predominantly binds to immune cells via Siglec-9,[11] a α2-3-linked sialic acid binding I-type lectin known to serve as an inhibitory immune cell receptor.[13–17] Siglec-9 is expressed on approximately 30–40% of the $CD16^{pos}/CD56^{dim}$ NK cell subsets, and in ovarian cancer patients these cells are double positive for sMUC16.[11] High expression of Siglec-9 is observed on >90% of the monocytes, and a correspondingly high level of sMUC16 binding is observed on these immune cells in ovarian cancer patients.

MUC16 is expressed by endometrial epithelial cells and also in the decidua.[18–21] Indeed, serum CA125 levels increase during pregnancy,[22] which is one of the reasons why serum CA125 cannot be used as an early diagnostic test exclusively for ovarian cancer. Considering our previous work on sMUC16 binding to PBMC and PFMC of ovarian cancer patients,[11,12] we investigated whether the mucin is also present on specific subsets of immune cells of pregnant women.

Our results indicate that the binding pattern of sMUC16 to NK cells and monocytes from peripheral blood of pregnant women closely matches the expression of Siglec-9 on these immune cells. Important differences were observed in the subsets of NK cells from normal pregnant women and preeclampsia patients that bind to sMUC16 and express Siglec-9. Data presented in this study lay the groundwork for future studies on the potential biological significance of sMUC16 binding to immune cells in healthy pregnant women and preeclampsia patients. In addition, differences in the binding patterns of sMUC16 to NK cells and the expression of Siglec-9 on these cells may also be exploited for the development of a novel diagnostic test for the detection of preeclampsia.

Methods

Cell Lines and Reagents

Siglec-9 expressing Jurkat cells were a kind gift from Dr. Jim Paulson (Scripps Research Institute, CA, USA). The Jurkat cells were cultured in RPMI-1640 media supplemented with 10% fetal calf serum. All other reagents were commercially obtained. ECC-1 and OV-CAR-3 cells were purchased from ATCC (Manassas, VA, USA) and were cultured in RPMI 1640 media containing 10% fetal bovine serum. Fluorophore-conjugated anti-CD14 (PerCP-CY5.5, clone: M5E2), CD3 (APC-Cy7, clone SK7), CD56 (Alexa 700, clone B159), CD16 (PE-Cy7), clone 3G8), CD19 (PE, clone HIB19), and Siglec-9 (CDw329; FITC, clone E10-286) were from BD Biosciences (San Jose, CA, USA), and secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). All other reagents were from Sigma (St Louis, MO, USA) or Invitrogen (Carlsbad, CA, USA).

Subjects

All subjects signed an informed consent, and the study was approved by the Institutional Review Boards of the University of Wisconsin-Madison and Meriter Hospital. The women were recruited at the time of admission to Labor and Delivery. Subjects were considered eligible controls if they had completed 37 weeks of gestation, had an uncomplicated prenatal course, and had no preexisting co-morbidities. Eligible preeclamptic subjects were identified using strict diagnostic criteria. Prenatal records were reviewed to ensure that there was no evidence of hypertension prior to 20 weeks of gestation. Hypertension was defined as systolic blood pressures of at least 140 mmHg or diastolic blood pressures of at least 90 mmHg two times measured 6 hr apart. The definition of proteinuria is the excretion of 300 mg of protein or greater in a 24-hr urine collection.[23] Urine dip analysis alone without confirmatory 24-hr urine protein excretion was not considered sufficient to enroll a subject into the study. Subjects were excluded from the study if they carried the diagnosis of chronic hypertension, diabetes, antiphospholipid lipid antibody syndrome, or systemic lupus erythematous. Subjects were also excluded if given the diagnosis of abruption or had meconium-stained amniotic fluid. Of the 26 women included in the study, there was one African American and all others were Caucasian. Table 1 shows the baseline characteristics of the groups. The groups showed significant difference in age as well as gestational age, which is expected when considering the disease process.

Isolation of Peripheral Blood Lymphocytes

Blood samples were obtained upon delivery from full-term uncomplicated pregnancies as well as preg-

Table I The baseline characteristics of the groups

Characteristics	Controls (n = 17)	Preeclamptics (n = 9)	P value
Age	33.45 (22–42)	27.4 (19–40)	0.0095
Body mass index (BMI)	32.45 (26–47)	37.2 (27–51)	0.0555
Gestational age at delivery (GA)	38.75 (37.2–41)	35.89 (28.1–38.3)	0.0009

nancies complicated by preeclampsia. Mononuclear cells were isolated under sterile conditions using Histopaque (Sigma Aldrich). The mononuclear layer was retained and washed once with PBS-BSA before being cryopreserved in 90% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) containing 10% dimethyl sulphoxide (DMSO). Serum samples were layered over Histopaque with the mononuclear layer being isolated and washed and cryopreserved.

Analysis of Siglec-9 and MUC16 on NK Cells by Flow Cytometry

Cryopreserved cells were thawed and washed (15 min at 300× ***g*** in PBS-BSA). Incubation of cells with primary and secondary antibodies was performed for 30 min on ice. After incubation with each antibody, cells were washed with PBS-BSA at 300× ***g*** for 10 min at 4°C. Cells were blocked with goat IgG prior to staining with anti-MUC16 antibody VK8. The cells were washed, and Allophycocyanin (APC)-conjugated goat anti-mouse (GAM) IgG antibody was added. The cells were then washed and incubated with mouse IgG for 20 min to bind any additional Fab sites on the GAM secondary. The cells were then incubated with a cocktail of fluorophore-conjugated antibodies to stain for CD3, CD45, CD56, CD16, and Siglec-9.[11,12]

After the final wash, cells were resuspended in approximately 300 μL of PBS-BSA. Immediately, prior to data acquisition on the LSRII flow cytometer (Beckton Dickinson, San Jose, CA, USA), DAPI, a viability indicator was applied to each sample. Each day, quality control procedures were run on the LSRII prior to data acquisition. Beckton Dickinson Cytometry Setup and Tracking (CST) beads were run daily to reproducibly set up the cytometer. Use of these beads allows for determination of cytometer baseline, the standard deviation of electronic noise, and assessment of photomultiplier tube (PMT) drift and adjusts cytometer settings to maximize population resolution in each detector. These measurements also verify that data are acquired within the linear range of each PMT. Spherotech Rainbow Beads, both mid-range and ultra, were run each day to determine that for a given PMT voltage, nearly identical target values [mean fluorescence intensity (MFI)] were obtained for each detector of interest. Setting the instrument up in this manner, with these controls, allowed for the negative controls from each patient for each day to give rise to comparable MFIs in each fluorescence channel. From here, compensation controls were generated and acquired for experiments carried out on individual days to adjust for spectral overlap between PMTs. Flow Jo software was used to analyze the flow cytometry data.

CA-125 Assay

Serum was separated, and the number of units of CA125 in the sample was determined by using the standard clinical radioimmunoassay (Abbott Axsym, Abbott Park, IL, USA). CA125 measurements were conducted in the clinical pathology laboratory of the University of Wisconsin-Madison Hospital and Clinics.

Immunoprecipitation of sMUC16 from Immune Cell Lysates

The PBMC's from healthy donor, ovarian cancer patient and OVCAR-3 ovarian cancer cell line were lysed in RIPA buffer containing protease inhibitors. After calculating the protein concentration (BCA), lysate equivalent to 500 μg of total protein was mixed with 100 μg of VK-8 antibody and tubes were rotated overnight at 4°C. Protein G-agarose beads (100 μL) were added to the mixture, and the tubes were rotated for 1 hr at room temperature. Suspensions were centrifuged (400 × ***g*** for 1 min), supernatants were removed, and the beads were washed three times with RIPA buffer (500 μL) containing protease inhibitors. Beads were then resuspended in 30 μL Laemmli buffer, boiled for 5 min, and loaded on 7.5% SDS–PAGE gel. Proteins from the gel were transferred to PVDF membranes, and MUC16 was detected using the VK-8 as the primary antibody and a HRP-conjugated goat anti-mouse as the secondary antibody.

MUC16 RT-PCR

OVCAR-3, ECC-1, and PBMC samples were homogenized in Trizol, and RNA was isolated according to the manufacturer's instructions (Invitrogen). The RNA was reverse transcribed into cDNA using Omniscript RT kit from Qiagen; Germantown, MD, USA (Cat.No.205111). The MUC16 and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) were amplified with the respective primers (MUC16 forward: 5′GCCTCTACCTTAACGGTTACAATGAA3′, reverse: 5′GGTACCCCATGGCTGTTGTG-3′; and GAPDH forward: GAGTCAACGGATTTGGTCGT, reverse: TTGA TTTTGGAGGGATCTCG) using fast cycling PCR kit from Qiagen as described in our earlier studies.[12,24] MUC 16 was amplified using the initial activation for 10 min at 95°C, followed by 35 cycles of 15 s at 95°C and 1 min at 60°C. The cycling conditions used for amplifying GAPDH were, initial activation at 95°C for 5min, followed by 30 cycles of the denaturation at 96°C for 5 s, annealing at 60°C for 5 s and extension at 68°C for 10 s, with final extension at 72°C for 1 min. GAPDH was used as an endogenous reference to determine the integrity of the mRNA in each sample. The PCR product was run on 2.5% agarose gel at 100 V for 2 hr, and bands were visualized using Flourchem8900 ultraviolet transilluminator (Alpha Innotech, San Leandro, CA, USA).

Statistics

The Flo Jo flow cytometry software was used to obtain the raw data on the percentage of immune cells positive for sMUC16, Siglec-9 and other immune markers. The data were plotted using the Graph Pad statistical software. Statistical significance of the data was determined using the nonparametric Mann–Whitney *U*-test.

Results

sMUC16 Binds to Immune Cells Isolated from the Peripheral Blood of Pregnant Women

Immune cells from healthy individuals and ovarian cancer patients do not express endogenous MUC16 but instead bind the mucin that is present in the serum and the PFMC of ovarian cancer patients.[11] Higher levels of sMUC16 (measured as CA125) are also observed in the serum of pregnant women as compared to the healthy non-pregnant cohort.[21,25] We therefore tested whether similar to our observation with immune cells from ovarian cancer patients, sMUC16 also binds to immune cells of pregnant women.

MUC16 was immunoprecipitated from the lysates of PBMCs isolated at the time of delivery from healthy pregnant women. However, this mucin was not detected in the immunoprecipitates from the lysates of healthy donor derived PBMCs (Fig. 1a). RT-PCR studies demonstrated that similar to ovarian cancer,[12] the PBMCs from healthy pregnant women did not express endogenous MUC16 (Fig. 1b).

We have previously shown that sMUC16 binds to immune cells via Siglec-9.[11] Binding of sMUC16 to Siglec-9 is rapid as shown by *in vitro* studies with Jurkat cells expressing this receptor (Fig. 2). The lack of MUC16 synthesis by immune cells of pregnant women suggested that similar to our observations in ovarian cancer, MUC16 is also captured on the surface of immune cells in pregnancy via Siglec-9. In subsequent experiments, we therefore simultaneously monitored sMUC16 binding and Siglec-9 expression on the immune cells from pregnant women and preeclampsia patients by using multi-color flow cytometry.

sMUC16 Binding Pattern to Immune Cell Subsets in Pregnancy

All of our flow cytometry experiments require careful standardization of the instruments with 2calibration

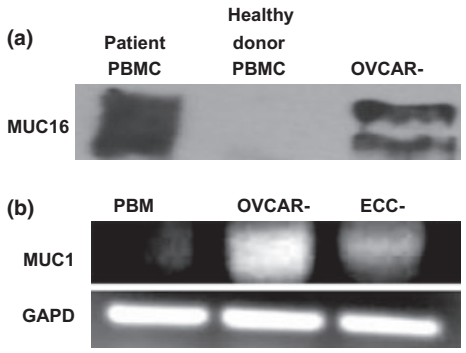


Fig. 1 sMUC16 is captured but not expressed by immune cells of pregnant women. (a) Peripheral blood mononuclear cells (PBMC) isolated from healthy pregnant women (lane 1) and a non-pregnant individual were lysed and immunoprecipitated with the anti-MUC16 antibody, VK8.[51] The immunoprecipitated material (5 μg/lane) was analyzed by western blotting, and VK8 was used for detection of the mucin. Lysate of ovarian cancer cell line OVCAR-3 was used as positive control (lane 3). (b) mRNA was isolated from PBMCs from women with healthy pregnancy, and MUC16 was detected by qPCR. mRNA from OVCAR-3 and ECC-1 cells were used as positive controls.

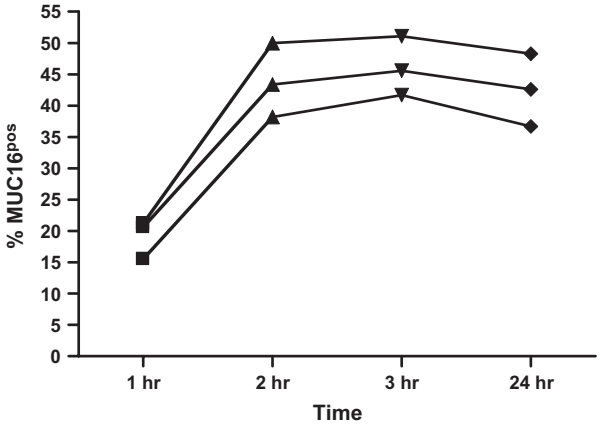


Fig. 2 Rapid binding of sMUC16 to immune cells via Siglec-9. Jurkat cells transfected with Siglec-9 were incubated with sMUC16 partially purified from the conditioned media of OVCAR-3 cells. Binding of the mucin to the Siglec-9 expressing Jurkat cells was determined by flow cytometry.

beads. We are therefore able to compare the binding pattern of sMUC16 and Siglec-9 expression in immune cells from pregnant subjects. Analysis of PBMC from healthy pregnant women indicated sMUC16 binding to B cells, NK cells, and monocytes (Fig. 3). In the case of NK cells, equal levels of the mucin were observed on the $CD16^{pos}/CD56^{dim}$ and $CD16^{neg}/CD56^{br}$ subsets (Fig. 3). The sMUC16 binding pattern in pregnancy samples matched the expression of Siglec-9 on the two NK cell subsets, further suggesting that Siglec-9 is the predominant receptor for sMUC16 on these immune cells (Fig. 3).

Low level of sMUC16 was detected on $CD3^{pos}$ T cells that have previously been shown to express only low levels of Siglec-9.[17] On the other hand, monocytes express high levels of Siglec-9 and were also strongly positive for sMUC16 (Fig. 3). In the case of B cells from pregnant women, only 10% of the cells were double positive for sMUC16 and Siglec-9 even though approximately 80% of the Siglec-9^{neg} B cells were positive for sMUC16 (Fig. 3). These data suggest that at least on the B cells, sMUC16 binding is Siglec-9-independent. Galectin-1 is another reported binding partner of MUC16.[26] We have not found any correlation between Galectin-1 expression on B cells and sMUC16 (data not shown).

NK Cell Bound sMUC16 is Detected Even at Low Serum CA125 Levels

Maximum serum CA125 levels are observed in the first trimester of pregnancy.[22,25] We therefore monitored serum CA125 levels and immune cell bound sMUC16 in three women before and at 9 and 18 weeks of pregnancy. A gradual increase in serum CA125 was observed at 9 weeks of pregnancy.[12] Continued monitoring of these three patients at 18 weeks of the pregnancy (same gestation as that monitored in our previous study[12]) showed a trend toward lower serum CA125 levels (Fig. 4). However, levels of sMUC16 bound to immune cells continued to show an upward trend at 18 weeks of pregnancy even as the serum CA125 levels decreased (Fig. 4).

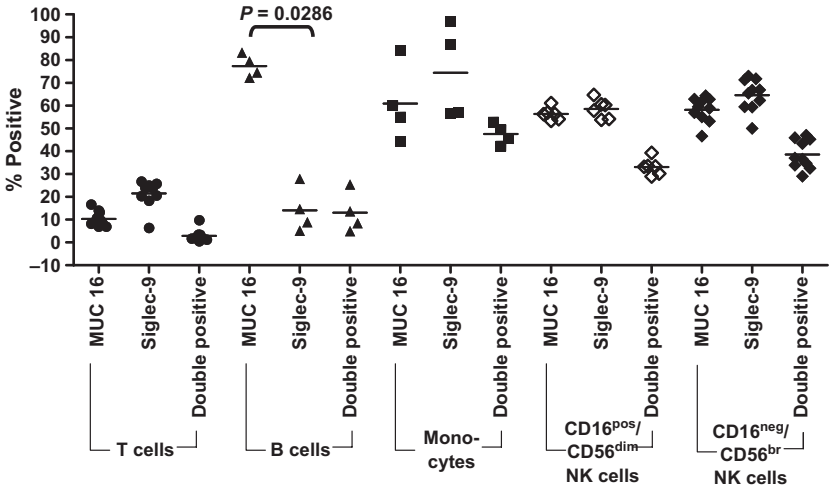
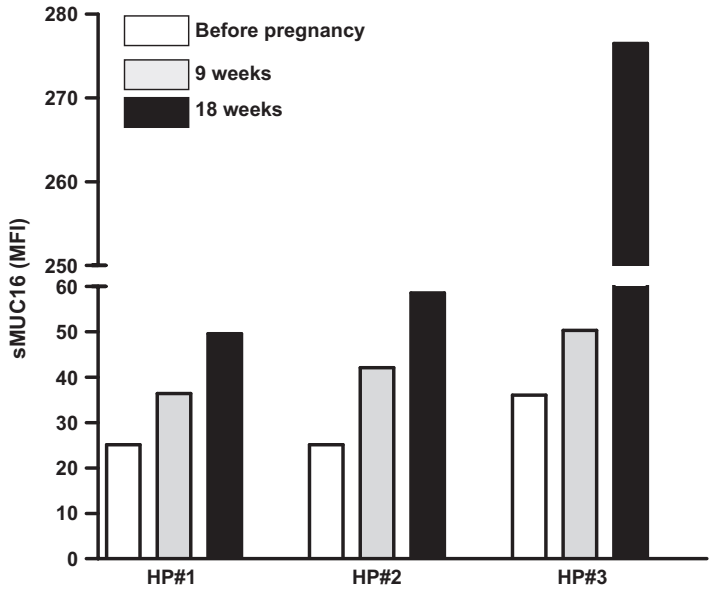


Fig. 3 sMUC16 predominantly binds to Siglec-9 expressing cells isolated from the peripheral blood of healthy pregnant women. sMUC16 binding to T cells, B cells, NK cells, and monocytes from peripheral blood mononuclear cells (PBMC) of healthy pregnant women was determined by flow cytometry. Siglec-9 positive events were gated, and the binding of sMUC16 to these immune cell subsets was determined. Immune cells from each patient were analyzed by flow cytometry in duplicate, and the data for each reading are shown.



	HP#1			HP#2			HP#3		
	Before pregnancy	9 weeks	18 weeks	Before pregnancy	9 weeks	18 weeks	Before pregnancy	9 weeks	18 weeks
CA125 (Units/mL)	<2	24	8	<2	40	17	<2	14	11

Fig. 4 Increasing levels of immune cell bound sMUC16 during pregnancy. Three healthy pregnant women (HP#1, HP2, and HP3) were analyzed for serum CA125 levels (units/mL shown below bar graph), and sMUC16 bound to peripheral blood immune cells (bar graph) isolated at weeks 9 and 18 of pregnancy. Immune cells from these three donors prior to their pregnancy were also available and were also tested for bound sMUC16. Bar graphs show the mean fluorescence intensity (MFI) for the total sMUC16 positive events in each sample used in this analysis.

MUC16 Binding to Immune Cells of Patients with Preeclampsia

Impairment of immune responses, especially those mediated by decidual NK cells, has been associated with the pathogenesis of preeclampsia.[27,28] Given the demonstrated immunomodulatory roles of sMUC16,[7,8] we next determined whether there were any differences in the binding pattern of this mucin to immune cells in preeclamptic patients compared with healthy pregnant women. First, we confirmed the results of previous studies[22,29,30] that the serum CA125 levels were not significantly different between healthy pregnant women and preeclampsia patients (Fig. 5a). Similarly, there was no statistically significant difference in the percentage of monocytes (Fig. 5b) and NK cells (data not shown) in the PBMC of healthy pregnant women and preeclampsia patients.

Next, we compared the binding of sMUC16 and expression of Siglec-9 on NK cells and monocytes from PBMC of preeclampsia patients and healthy pregnant subjects. Samples from healthy pregnant women described in Table 1 were analyzed in these experiments but did not include samples from patients who were already studied for experiments described in Fig. 3. The preeclampsia cohort included patients with both mild as well as severe disease, however a subgroup analysis was not performed in this initial study.

Similar to our observations in healthy pregnant women, sMUC16 was also detected on NK cells and monocytes isolated from the PBMC of patients with preeclampsia (Figs 6 and 7). The pattern of sMUC16 binding was distinct between the NK cell subsets of healthy pregnant women and preeclamptic women. Siglec-9 expression was comparable on the $CD16^{pos}/CD56^{dim}$ and $CD16^{neg}/CD56^{br}$ NK cells of the healthy pregnant women and the preeclampsia patients (Fig. 6a,b). However, a statistically significant increase in sMUC16 binding was observed on the $CD16^{pos}/CD56^{dim}$ and $CD16^{neg}/CD56^{br}$ NK cells of preeclampsia patients as compared to the healthy pregnant group (Fig. 6a,b). Significant differences were also observed in the case of monocytes isolated from healthy pregnant women and patients with preeclampsia. Increased sMUC16 binding was observed on monocytes of preeclampsia patients (Fig. 7). A trend for increased expression of Siglec-9 on the monocytes of preeclampsia patients was observed. However, the differences in expression levels of Siglec-9 between the two cohorts were not statistically significant ($P = 0.649$; Fig. 7).

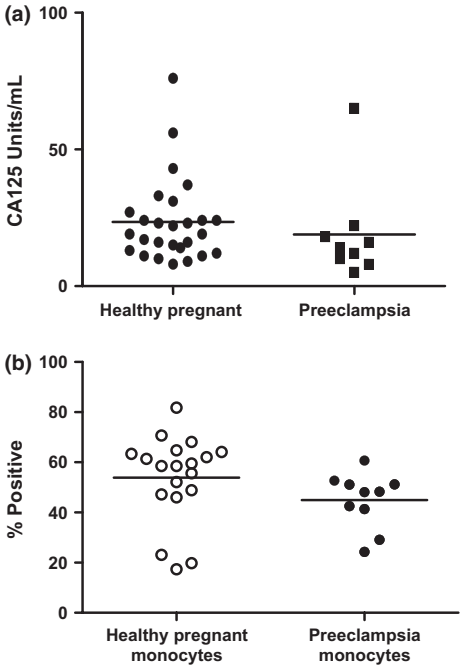


Fig. 5 Healthy pregnant women and preeclampsia patients have comparable levels of serum CA125 and monocytes. (a) Serum samples were drawn from healthy pregnant (n = 27) and preeclampsia patients (n = 9) and CA125 levels in each sample were determined using a clinical assay. (b) Healthy pregnant women and preeclampsia patients have comparable levels of peripheral blood monocytes. Comparable levels of immune cells were present in the peripheral blood of healthy pregnant women and preeclampsia patients. Representative data for peripheral blood monocytes are shown here. Data show the total number of $CD3^{neg}/CD16^{pos}/CD14^{pos}$ monocyte events as determined by flow cytometry in each blood sample.

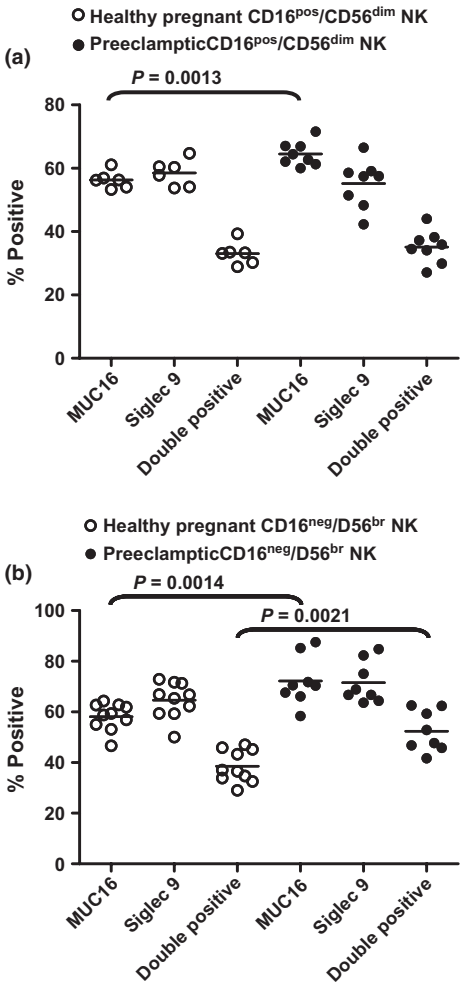


Fig. 6 Differential binding to sMUC16 to NK cell subsets of preeclamptic patients. Peripheral blood mononuclear cells isolated from healthy pregnant women and preeclampsia patients at the time of delivery were subjected to multi-color flow cytometry. The (a) $CD16^{pos}/CD56^{dim}$ and (b) $CD16^{neg}/CD56^{br}$ NK cell subsets were identified as described in our previous work.[11,12] sMUC16 was detected using the VK-8 antibody, and percentage of sMUC16 and Siglec-9 positive cells were identified. The flow cytometer was calibrated using fluorescent bead standards allowing us to compare the data obtained from all samples from experiments conducted on different days. Duplicate readings for each patient are shown.

Discussion

Mucins are high–molecular weight glycoproteins that exhibit important biological roles via their carbohydrate as well as protein epitopes.[10,31] Probably the best characterized roles of the protein epitopes of mucins are the importance of the C-terminal domains of mucins such as MUC1 and MUC4 in intracellular signaling.[32] Oligosaccharides attached to the extracellular domains of mucins have also been shown to interact with different lectin receptors and influencing functions such as cell–cell adhesion and regulating immune responses.[33–35] MUC16 is, to date, the largest mucin identified in the human genome and similar to other mucins of its class plays a major biological role in normal as well as pathologic tissues via its extensive N-linked and O-linked oligosaccharide chains and its approximately 24,000 amino acid protein backbone.[7,10,36–38]

Highlights of the biological activities of MUC16 reported at this point include its role in (i) facilitating ovarian tumor metastasis via its interactions with mesothelin, a GPI-anchored glycoprotein expressed on mesothelial cells and cancer cells,[37–39] (ii) allowing ovarian tumor cells to escape NK cell immune synapse

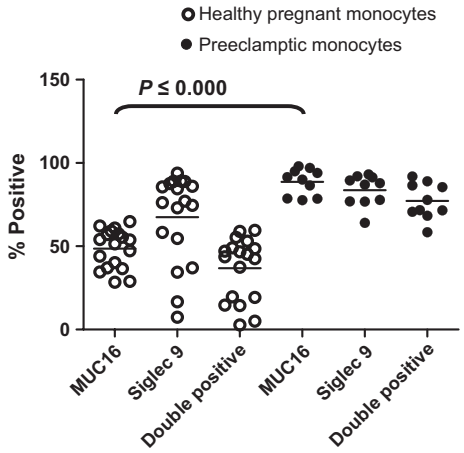


Fig. 7 Differential binding to sMUC16 to monocytes of preeclamptic patients. Peripheral blood mononuclear cells isolated from healthy pregnant women and preeclampsia patients at the time of delivery were subjected to multi-color flow cytometry. Monocytes were identified as described[11,12] and percentage of sMUC16 (detected using VK-8 antibody) and Siglec-9 positive events were identified. The flow cytometer was calibrated using fluorescent bead standards allowing us to compare the data obtained from all samples from experiments conducted on different days. Duplicate readings for each patient are shown.

formation and directly inhibiting the ability of NK cells to cytolyse cancer targets,[7,8] (iii) imparting resistance to chemotherapy in ovarian cancer cells,[40] (iv) contributing to cell survival in cancer cells,[41,42] and (v) serving as a barrier to adhesion of trophoblasts to the endometrial epithelium and of bacteria to the corneal epithelia.[43] Considering this background, our observations that sMUC16 binds to immune cells during pregnancy and shows higher level of binding to the $CD16^{neg}/CD56^{br}$ NK cell subset and monocytes from preeclamptic patients are likely to have important physiologic consequences. First, sMUC16 binding to monocytes and $CD16^{pos}/CD56^{dim}$ NK cells is likely to inhibit the cytolytic activities of these immune cells, as shown in our previous study thereby ensuring suppression of maternal cytotoxic immune responses against the fetal tissues.[8]

Second, the $CD16^{neg}/CD56^{br}$ NK cells exhibit low cytotoxicity and instead are high producer of cytokines.[44] This NK cell subset has been conclusively implicated as performing a pro-fetal growth function by expressing angiogenic cytokines that enhance trophoblastic invasion of the uterine spiral arterioles.[28]

Recent data emerging from experiments with monocytic cell lines indicate that engagement of Siglec-9 induces these cells to increase expression of the immunosuppressive cytokine IL-10.[45] This cytokine is known to promote placental angiogenesis. Thus, sMUC16 binding to monocytes and NK cells may likely promote fetal development. Increased binding of sMUC16 to $CD16^{neg}/CD56^{br}$ NK cells and monocytes in preeclampsia may be a corrective maneuver to increase VEGF levels to promote angiogenesis in pregnancy.

Preeclampsia is a hypertensive disorder unique to pregnancy and affects 2–7% of pregnancies.[23,46] It is a major cause of both maternal and fetal morbidity and mortality. Considered a leading cause of iatrogenic prematurity, preeclampsia is characterized by poor perfusion to multiple vital fetal organs, which is reversed upon delivery. A primary pathological feature of preeclampsia is insufficient invasion of the spiral arterioles by the fetal trophoblast with subsequent widespread systemic endothelial dysfunction. $CD16^{neg}/CD56^{br}$ NK cells present in the decidua are implicated in successful trophoblastic invasion of the uterine spiral arterioles and the regulation of uterine blood flow.[28]

Maternal hypertension is the primary mode of detection of preeclampsia. Increase in maternal blood pressure, however, occurs at a later stage in pregnancy. Attempts are therefore underway to identify biomarkers that can detect the onset of preeclampsia at an early stage.[47–49] Past reports have studied potential increases in the serum levels of CA125 and demonstrated no significant change in the levels of this marker between women with healthy pregnancies and those with preeclampsia.[22,29] The studies presented here suggest a novel method for the identification of biomarkers that can predict preeclampsia. Based on this method, we predict that binding of sMUC16 bound to circulating $CD16^{neg}/CD56^{br}$ NK cells and monocytes above a threshold value may serve as an important method for detection and monitoring of preeclampsia. Additionally, because sMUC16 also binds to $CD16^{pos}/CD56^{dim}$ NK cells and monocytes in ovarian cancer patients, monitoring the levels of this mucin on immune cells may also prove important in detecting and monitoring ovarian cancer.[11,12] Immune cell–bound sMUC16 provides an advantage over the conventional serum CA125 assay as it does not primarily rely on the absolute concentration of the biomarker in the serum, a major obstacle because the concentration of the biomarker may vary based on its rapid turnover by the liver,[50] but instead relies

on the measurement of the captured antigen on specific immune cell subsets.

Analysis of a large cohort of healthy donors, healthy pregnant women, and patients with ovarian cancer or preeclampsia will be necessary to establish threshold values for sMUC16 binding to NK cell subsets and monocytes to distinguish between healthy pregnant women and patients with preeclampsia. These studies are currently underway in our laboratories. Sequential samples obtained from ovarian cancer patients undergoing chemotherapy and pregnant diabetic women (a high risk group for preeclampsia) are being monitored for sMUC16 binding and Siglec-9 expression on immune cell subsets. These studies will be very useful in providing a profile of sMUC16 binding and Siglec-9 expression on immune cells and its potential in early identification and monitoring of ovarian cancer and preeclampsia.

Acknowledgements

VK-8 antibody used in these experiments was a generous gift from Dr. Beatrice Yin, Memorial Sloan Kettering. Funding for this research was provided by grants from, Congressionally Directed Medical Research Program of the Department of Defense (W81XWH-11-1-0181), UW School of Medicine and Public Health Wisconsin Partnership Program, The Robert Draper Technology Innovation Fund, a charitable donation from Jean McKenzie, Wisconsin Alliance for Ovarian Cancer, Department of Obstetrics and Gynecology, and National Institutes of Health (R21CA143616) grant to MSP and JPC. This investigation was supported by the National Institutes of Health under Ruth L. Kirschstein National Research Service Award 5T32HD041921 to C.T.T. and J.A.A.G. C.T.T. is also the recipient of the Centennial Scholars Research grant and is BIRCWH scholar. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. We are deeply grateful to Kathy Schell for her advice and help and acknowledge the support provided by the University of Wisconsin Comprehensive Cancer Centers Flow Cytometry facility, which is supported by a core grant (CA14520) from the National Institutes of Health.

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